Dissection of Human Papillomavirus E6 and E7 Function in Transgenic Mouse Models of Cervical Carcinogenesis

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ABSTRACT

Human cervix cancer is caused by high-risk human papillomaviruses encoding E6 and E7 oncoproteins, each of which alter function of distinct targets regulating the cell cycle, apoptosis, and differentiation. Here we determined the molecular contribution of E6 or E7 to neoplastic progression and malignant growth in a transgenic mouse model of cervical carcinogenesis. E7 increased proliferation and centrosome copy number, and produced progression to multifocal microinvasive cervical cancers. E6 elevated centrosome copy number and eliminated detectable p53 protein, but did not produce neoplasia or cancer. E6 plus E7 additionally elevated centrosome copy number and created large, extensively invasive cancers. Centrosome copy number increases and p53 loss likely contributed to malignant growth; however, dysregulated proliferation and differentiation were required for carcinogenic progression.

INTRODUCTION

Cervical cancer, a worldwide health problem, has been directly linked to genital infection by HPV. HPV and HPV DNA and viral gene expression has been detected in nearly all cervical malignancies (1, 2). HPVs are mucosal-trophic viruses infecting basal squamous epithelial cells, with the productive phase of the viral life cycle elaborated in the upper squamous epithelial cell layers. Most HPV infections are transient, but in the minority of patients persistent viral disease localizes in basal squamous cervical epithelial cells of the cervix, and underlies neoplastic progression and emergence of invasive malignancies (3).

There are >100 types of HPVs, but only a subset of “high-risk” viral types induce cervical carcinogenesis. HPVs contain two genes in their early region, E6 and E7, and high-risk viral types encode E6 and E7 oncoproteins with an enhanced affinity for host cellular targets (1, 4). Whereas transcriptional activity has been associated with both E6 and E7, these oncoproteins are not DNA binding transcription factors, the major thrust of investigation has centered on protein-protein interactions with cellular targets (5–7). In many instances, association of the cellular target with an HPV oncoprotein leads to target protein destabilization and ultimate destruction (8–10). In other instances, viral oncoprotein binding may activate the host cellular protein (11). Several studies support a functional role for the E6 and E7 oncoproteins in tumorigenesis. Both oncoproteins are expressed consistently in cervical cancer tissues obtained from patients (2). Cell transfection of high-risk HPV16 or 18 E6 and E7 oncoproteins forms established cell lines and immortilizes primary cells (12–15). Ubiquitous or targeted expression of E6 and E7 has been shown to produce benign tumors or cancers in transgenic mice (16–21). The potential importance of E6 and E7 function in cervical carcinogenesis has also spawned vaccine development targeting viral oncoprotein epitopes (22–25). These vaccine efforts are a parallel approach to prophylaxis based on immunization against viral capsid antigens (26).

Rational drug design is also being applied to target the enzymatic functions of E6 or more recently E7 (27–29). Thus, the importance of HPV oncoproteins for viral infection, carcinogenesis, and potential therapy mandates a complete understanding of their molecular, cellular, and tissue biology in cervical epithelial cells.

However, despite insights into viral oncoprotein function, the discrete biology of the individual E6 and E7 oncoproteins in cervical epithelial cells is unclear. Therefore, we combined our expertise in the induction of cervix cancer in HPV transgenic mice by chronic estrogen administration (30, 31) with transgenic mice engineered to express either HPV E6 or E7 oncoprotein individually (32), to determine the discrete effects of each HPV oncoprotein in cervical carcinogenesis. Surprisingly, E7 alone was sufficient to produce both high-grade cervical dysplasia and invasive cervical malignancies. In mice engineered for expression of E6 oncoprotein alone, only low-grade cervical dysplasia was evident without additional neoplastic progression within the 6-month treatment interval of the study. Coexpression of E6 and E7 in double-transgenic mice revealed that E6 modulated the malignant phenotype produced by E7, in that cervical cancers were larger and more extensive. As such, this study demonstrates the potent cocarcinogenic activity of the HPV16 E7 oncoprotein with estrogen in the mouse cervix. HPV16 E6 appears to have predominant efficacy as a modulator of E7 estrogen cervical cocarcinogenesis.

MATERIALS AND METHODS

Transgenic Mice. The K14-HPV16 (19), K14-E7 (32), and K14-E6 (33) transgenic mice have been described previously. K14-HPV16 have been backcrossed in the FVB/n background for 40 generations, maintained, and used as heterozygotes in these experiments. Mice expressing either E7 or E6 oncoproteins were transgenic with constructs containing only the overlapping HPV16 E6 and E7 ORFs spanning nucleotides 79–883. K14-E6ttt/E7 (designated as K14-E7) transgenic mice have a ttt in the E6 gene precluding E6 expression (32). K14-E7 transgenic mice were maintained and used as transgenic heterozygotes. K14-E6/E7tt (designated as K14-E6) transgenic mice contain a ttt in the E7 region and were used as transgenic homozygotes (33). K14-E7 transgenic mice and K14-E6 transgenic mice were created and maintained in the FVB/n inbred strain. K14-E5/E7 mice were derived by intercrossing heterozygous K14-E7 with homozygous K14-E6 mice generating double-transgenic K14-E6/E7 heterozygous progeny. All of the mice were housed in a pathogen-free barrier facility, and all of the experiments and procedures were approved by the University of California San Francisco Committee on Animal Research.

Hormone Treatment. One-month virgin female transgenic and nontransgenic mice were anesthetized with halothane, s.c. implanted in the dorsal skin with continuous release pellets delivering 0.05 mg 17β-estradiol over 60 days (Innovative Research of America, Sarasota, FL). Mice were treated with hormone for 1, 3, or 6 months; the longer treatment times required an additional insertion of one or two pellets (31). Groups of 4–7 transgenic and nontransgenic mice were treated for 1 and 3 months. Larger groups comprising 20 (K14-E6, K14-E7, K14-E6/E7, and nontransgenic) or 40 (K14-HPV16)
transgenic mice were treated with 17β-estradiol for 6 months. Data from the K14-HPV16 transgenic mice were pooled from two concurrent studies.

Tissue Procurement, Histopathological Analysis, and Determination of Cancer Size. Mice were anesthetized with 2.5% Avertin and perfused through the aorta with 3.75% paraformaldehyde. The entire reproductive tract including parametrial, retroperitoneal soft tissue and lymph nodes were dissected, placed in an embedding cassette (Fisher) with one sponge for compression, and postfixed in 3.75% paraformaldehyde overnight at 4°C. In-cassette postfixation produced a linear reproductive tract facilitating subsequent sectioning of the entire organ in one plane (Fig. 1). The posterior vaginal wall was removed leaving an intact rim of vulvar tissue, and a 3–5 mm end piece of plastic pipette tip was inserted into the vaginal opening to keep the vagina open during subsequent processing steps. Tissues were washed in 1 × PBS, and dehydrated through graded alcohols and xylene using a Leica TP1050 vacuum tissue processor (Leica Microsystems, Inc., Bannockburn, IL). After pipette tip removal, the reproductive tract was embedded with the cut vaginal wall surface oriented downward. The entire reproductive tract was serially sectioned, and 10–15 sections were collected at 75 μm intervals for H&E staining and immunohistochemistry.

To determine cancer size, malignancies from reproductive tracts of transgenic mice treated with estrogen for 6 months were visually screened histologically and classified as “small” or “large.” Each large cancer was then measured using the Axiosvision 4.0 software package on images acquired from a Zeiss Axioplan 2 imaging microscope with an AxioCam color-mosaic CCD sensor. Images were acquired at 14-bitdepth and the programmable 1300 × 1030 pixel resolution for each color channel. Pixel conversion to microns was done by calibrating each objective with a stage micrometer. Each large cancer measured >2000 μm².

HPV16 E6/E7 mRNA Transgene Expression. Transgenic (n = 6 for each genotype; K14-HPV16, K14-E7, and K14-E6 transgenic mice) and nontransgenic (n = 4) mice were treated with 17β-estradiol for 1 month. After sacrifice by cervical dislocation and thoracotomy, the uterus, cervix, and vagina were rapidly removed. The uterine horns were separated and discarded from the cervix, vagina, and vulva, which were maintained as an intact tissue block (see cervical-vaginal region, Fig. 1). This distal reproductive tract block was snap frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted after a 90 s homogenization in TRIZol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. DNA was removed using AMBION DNA free kit (Ambion, Austin, TX). RNA samples were quantified by UV spectroscopy, and 500 ng of total RNA was used for reverse transcription, as described previously (16). After inactivation of reverse transcriptase by heating at 95°C for 5 min, transgene E6/E7 expression was determined by real-time TaqMan RT-PCR (34, 35) using PCR primers 5’ and 3’ to the E6+ splice site and a TaqMan probe specific for the 3’ splice acceptor side of the E6+ splice site. This primer and TaqMan probe set detected both full-length E6 and E6+ mRNA. Primer and TaqMan probe sequences were: E6 forward, (5’) GCA-CAGAGCTGCAAACACACTATACA; E6 reverse, (5’) GGTTTITGTCCAGGTGTCTTTGTGC; TaqMan probe, 6-carboxyfluorescein-TGGGACGTTGAGGTTGATTTAAACTGTCAAAGC-6-carboxy-tetramethyl-rhodamine-3’ (Integrated DNA Technologies, Coralville, IA). PCR was conducted in triplicate, on an ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA), in 50-μl reaction volumes containing: 1× PCR buffer, 5.5 mM MgCl₂, 0.9 μM of each primer, 200 μM dNTPs, 200 nM of probe, and 0.025 units/μl Taq Gold (Applied Biosystems). PCR cycling conditions consisted of 95°C for 15 min, followed by 40 cycles of 95°C for 30 s and 60°C for 60 s. Using histogram 3.3A as an endogenous control gene, relative expression levels for E6 ORF expression were calculated as 2^(-ΔΔCt; E6 -Ct histogram 3.3A) as described previously (35).

Determination of Reproductive Tract Proliferation Using BrdUrd Incorporation. Labeling of S phase cells by BrdUrd was performed as described previously (19, 36). An intermediate magnification image of the entire cervical-lower uterine transformation zone (see box, Fig. 1) was captured, and all of the S phase epithelial cells within this region were counted. The entire squamous epithelium of the transformation zone, from the squamo-columnar junction to the opening of the cervical canal, but not including the canal, was counted on five to six adjacent ×10 fields. Analysis for the number of BrdUrd-positive cells was conducted on 4–7 animals per genotype and time point.

p53 Immunohistochemistry. Five μm paraffin-embedded sections were processed as described above and endogenous peroxidase activity quenched with 3% H₂O₂ in methanol for 30 min, followed by antigen retrieval (Retrievit pH 2; Biogenex, San Ramon, CA) in a 95°C water bath for 35 min. After room temperature equilibration, sections were washed in PBS and blocked at 5% albumin/5% goat serum in PBS for 30 min. An avidin and biotin block was performed for 15 min each per the manufacturer’s instructions (Vector Laboratories). The p53 antibody (CM 5 clone; Novacasta) was applied overnight at 4°C and diluted 1:200 in 5% albumin:5% goat blocking kit; Vector Laboratories). The p53 antibody (CM 5 clone; Novacasta) was applied overnight at 4°C and diluted 1:200 in 5% albumin/5% goat serum. After incubation with a biotinylated goat-antirabbit (1:200; Vector Laboratories) antibody for 45 min, antibody detection and visualization was performed by sequential applications of the Vector Elite ABC kit, and the chromagen 3,3 diaminobenzadine (Sigma). Sections were counterstained in Gills #1 Hematoxylin (Sigma).

Determination of Apoptosis. For analysis of DNA fragmentation in the squamous epithelium of reproductive tracts, the Trevigen DermaTacs Apoptag kit (Trevigen, Gaithersburg, MD) was used, which incorporates BrdUrd into sites of double-strand DNA breakage. The experiments were performed according to the manufacturer’s instructions, except that reagent incubation times were extended, in addition to a less stringent blocking for endogenous peroxidase activity. Briefly, sections were digested with proteinase K (1:50) for 15 min, followed by endogenous peroxidase quenching in 1% H₂O₂ in PBS for 5 min. After equilibration in 1 × terminal deoxynucleotidyltransferase buffer, sections were incubated at ambient temperature for 1 h each with terminal deoxynucleotidyltransferase labeling mix and then the anti-BrdUrd antibody. Sections were subsequently incubated with peroxidase-conjugated streptavidin for 20 min, developed with Tacs blue label for 15 min, and counterstained for 10 min using Counterstain Red C (DermaTacs kit).

Determination of Centrosome Copy Number. Formalin-fixed, paraffin-embedded 5-μm tissue sections were deparaffinized in xylene for 30 min, rehydrated in alcohols and water as described above, and boiled in 10 μm

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**Fig. 1.** A composite H&E image of an entire K14-HPV transgenic reproductive tract treated with estrogen for 6 months. Division of the reproductive tract into anatomical regions that were comparatively analyzed for histopathology, DNA synthesis, and mRNA expression levels. Acquisition of a reproductive tract tissue section as displayed here requires special tissue processing techniques as detailed in “Materials and Methods.” Precise tissue orientation and step sectioning facilitates identification, localization, and assessment of the degree of malignant stromal invasion in each transgenic animal. Here a squamous carcinoma is present at the transformation zone (see inset).
citrate buffer (pH 6.0) for 30 min in a microwave oven. After a room temperature dH2O wash, sections were treated with Digest-All 3 (Zymed) for 10 min. Washed sections were incubated with 1:50 dilution of a polyclonal rabbit antiperoxidase. Antibody detection was performed using a 1:1,000 dilution of a rhodamine red-labeled donkey antirabbit antibody (Jackson Immunoresearch) for 2 h at 37°C. Sections were counterstained and mounted with 4',6-diamidino-2-phenylindole-Vectashield (Vector Laboratories). Cells were analyzed using a Leica DMLB Epifluorescence microscope equipped with a Sony DKC5000 digital camera system.

**Image and Statistical Analysis.** Images were captured using a SPOT CCD camera (Diagnostic Instruments, Sterling Heights, MI) attached to a LEICA DMRXA microscope. Figures were composed using Adobe Photoshop 6.0 (San Jose, CA). Numerical data are presented as the mean ± SD, and were analyzed statistically by ANOVA, two-tailed unpaired Student’s t test and χ² analysis, where appropriate.

**RESULTS**

**Estrogen Induction of Cervix Cancers According to Expression of Individual HPV ORFs.** To determine the discrete biology of the HPV16 E6 or E7 ORFs in mouse cervical carcinogenesis, K14-E7 and K14-E6 transgenic mice (see “Materials and Methods”) were treated with our protocol for induction of squamous carcinomas of the cervix and vagina by chronic estrogen treatment (31). Concurrent and sequential positive controls were K14-HPV16-transgenic mice containing the entire early region, and negative control, nontransgenic FVB/n littermates. The original studies of the p1427 and p1466 constructs, containing tils in either the E6 or E7 ORFs, respectively, demonstrated that these mutations were both molecularly and biologically tight, and lacked expression of attenuated E6 or E7 functions (15). Subsequent studies of transfected cells (37) and transgenic mice in which these expression of these constructs was targeted to lens epithelium (38), or basal epidermal keratinocytes (32, 33, 39, 40), also validated the discrete E6 or E7 functions encoded by each of these constructs. Strikingly, 80% of the estrogen-treated K14-E7 transgenic mouse developed reproductive tract carcinoma (Table 1). With the exception of one uterine adenocarcinoma, all of the reproductive tract cancers in the K14-E7-transgenic mice originated in squamous epithelium (see next paragraph). Similar to previous work, 83% of K14-HPV16-transgenic mice developed reproductive tract squamous cancer (Table 1; Refs. 30, 31). In stark contrast, none of the K14-E6 or nontransgenic mice developed reproductive tract malignancies.

Despite a similar incidence, there were significant differences in reproductive tract location, quantity, size, and growth characteristics of malignancies arising in K14-E7 versus K14-HPV transgenic mice (Table 1). To determine a distinct biology for each HPV transgenic genotype, the complete reproductive tract from ovaries to vulva was harvested, embedded, cut, and stained in one tissue plane, and the entire organ was analytically divided into discrete anatomical regions (Fig. 1). Reproductive tract squamous carcinomas in K14-E7 transgenic mice were dispersed throughout the cervico-vaginal epithelium, with a biased incidence favoring localization in the lower reproductive tract squamous epithelium (vulva, vagina, and outer cervix) compared with the cervical-uterine transformation zone at the junction of the upper cervix and lower uterus (Table 1; Fig. 1). In contrast, reproductive tract malignancy in K14-HPV16-transgenic mice was more frequently detected at the cervical transformation zone (Table 1). The number of mice with multiple cancers and the mean number of reproductive tract cancers per mouse were greater in K14-E7 compared with K14-HPV16-transgenic mice (Table 1). However, cervical cancer size was significantly less in the K14-E7 compared with either K14-HPV16 or K14-E6-transgenic counterparts (Table 1).

**Development of a Histopathological Grading System for Transgenic Mouse Cervical Squamous Carcinogenesis.** The marked discrepancy in the extent of carcinogenic progression in estrogen-treated K14-E7 versus K14-E6-transgenic mice motivated us to determine differences in histological progression according to genotype. Therefore, we developed a system to determine the degree of dysplastic or carcinogenic progression in the mouse cervix, initially using estrogen-treated K14-HPV16 transgenic mice in which histopathological progression to cervical malignancy was first demonstrated (Refs. 30, 31; Fig. 2). This grading system was based on the established criteria for classification of human cervical neoplasia or malignancy accounting for differences between the mouse model and patients. One major difference was that high-grade dysplastic mouse cervical epithelium always retained multiple suprabasal cell layers with largely intact terminal differentiation. In contrast, high-grade dysplasia or CIS in the human consisted of replacement of the entire extent of squamous epithelium by immature basal-type squamous cells, each of which are almost entirely composed of enlarged, anaplastic nuclei with scant cytoplasm (41). As such, the grade of neoplastic progression in mouse squamous epithelium was determined by the increase in nuclear:cytoplasmic ratio in individual squamous epithelial cells, the frequency of such cells within the squamous epithelium, and the morphology of the interface between squamous epithelium and the underlying vaginal or cervical stroma.

Using this mouse cervical neoplasia grading system, CIN-I consisted of a 2-fold increase in the basal/basaloid cell layers of cervical and vaginal squamous epithelia of transgenic compared with estrogen-treated nontransgenic mice (Fig. 2, compare panels 1 and 2). Interstratified in the basal and immediate suprabasal cell layers were individual cells with an increased nuclear:cytoplasmic ratio and nuclear atypia (Fig. 2, black arrowhead). CIN II lesions contained cells with

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean no. of cancers</th>
<th>Incidence of transformation zone cancer (%)</th>
<th>Incidence of mice with cancers &gt;2000 µm²</th>
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</thead>
<tbody>
<tr>
<td>K14-HPV</td>
<td>2.0 ± 1.1d²</td>
<td>50c</td>
<td>46c</td>
</tr>
<tr>
<td>K14-E6:E7</td>
<td>2.8 ± 1.4</td>
<td>40c</td>
<td>26c</td>
</tr>
<tr>
<td>K14-E7</td>
<td>3.8 ± 2.2</td>
<td>25c</td>
<td>6</td>
</tr>
<tr>
<td>K14-E6</td>
<td>0</td>
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</table>

- All cancers were squamous carcinomas, except for one uterine adenocarcinoma in the K14-E7 group.
- Denominator derived from previous column.
- Mean number of cancers per mouse with cancer.
- Compared to E7 mice P < 0.05.
- Homozygous transgenic mice.
applied our CIN grading system to determine the extent of neoplastic biology of E6 and E7 functions in the estrogen-treated cervix, we both quantified and determined the squamous epithelial growth also appeared to distinctively vary according to which HPV ORFs were expressed in the estrogen-treated cervix. Cervical and vaginal squamous carcinomas in K14-E7 transgenic mice were microinvasive, small, and multifocal (Fig. 3B, panel 5). Cervical malignancies in K14-E6:E7 and K14-HPV16 transgenic mice consisted of multiple nests of invasive squamous cells dispersed over a large area of cervical stromal tissue (Fig. 3B, panel 6). The distinctive biological differences in cancer growth in transgenic mice expressing E7 versus both E6 and E7 support a role for E6 as a late-stage progression factor for cervical cancer in this model.

Effect of Each HPV ORF on Reproductive Tract Squamous Cell Proliferation at Specific Stages of Carcinogenic Progression. One explanation for the differential extent of histological progression in K14-E7 compared with K14-E6 transgenic mice could be lack of induction of proliferation. To precisely determine the temporal and spatial effects of each HPV oncogene on cell proliferation in the cervix, we both quantified and determined the squamous epithelial distribution of BrdUrd-labeled cells in the entire cervical transformation zone after 1, 3, and 6 months of estrogen treatment (Fig. 4, A and B). The mean number of S phase squamous epithelial cells progressively increased at each time point in K14-HPV16 and K14-E7 compared with either K14-E6 transgenic mice or nontransgenic controls (Fig. 4A). Moreover, S phase cells were present in multiple cell layers in the squamous epithelia of both K14-E7 (Fig. 4B, panel 5) and K14-HPV16 (Fig. 4B, panel 7) transgenic mice, particularly in papillary projection of squamous epithelium into the cervical (and vaginal) stroma. In contrast, BrdUrd-positive cells were restricted to the basal and immediate suprabasal cell layers in cervical squamous epithelium of K14-E6 transgenic mice and nontransgenic controls (Fig. 4B, panels 1 and 2).

Analysis of the ear skin from the same transgenic mice from which reproductive tracts were harvested demonstrated marked differences, particularly in K14-E6 transgenic mice, compared that of the cervix. Each genotype of HPV transgenic mouse displayed numerous S phase cells in multiple epidermal cell layers. Moreover, BrdUrd-positive cells were present in the upper granular epidermal cell layers, a region restricted to nonreplicating, terminally differentiated keratinocytes in nontransgenic controls. These data are consistent with prior work (32, 33, 43), and likely reflect the ability of either the E6 or E7 oncoproteins to “uncouple” proliferation from terminal differentiation in epidermal keratinocytes (Ref. 44; see “Discussion”). These data highlight the discordant biology of the individual HPV oncoproteins in skin and cervix. In particular, E6 has no effect on the distribution of proliferating and differentiating cells in the estrogen-primed cervix, whereas additional increases in nuclear size, degree of anaplasia, frequency, and distribution of dysplastic cells in the suprapascal layers of the squamous epithelium (Fig. 2, panel 3). Moreover, the basal aspect of the squamous epithelium was thrown into papillary folds projecting into the underlying vaginal or cervical stroma (Fig. 2, panel 3). CIN III lesions contained abundant anaplastic cells, some with pronounced increases in nuclear size (Fig. 2, panel 4). CIS demonstrated a pronounced degree of remodeling and undulation of the epithelial-stromal border (Fig. 2, panel 5), and most of the cellular features of well-differentiated squamous carcinoma, with retention of an intact basement membrane without evidence of microinvasion on serial sections. Invasive, well-differentiated squamous cancers consisted of invading nests of cancer cells arranged in part in keratin pearls (Ref. 42; Fig. 2, panel 6). In contrast with transgenic mice, continuous estrogen treatment for 6 months in nontransgenic mice produced squamous epithelial hyperplasia similar to persistent estrus, with an increase in the number of squamous epithelial cell layers, occasional basal cell mitotic figures, and preservation of differentiated suprapascal keratinocytes (Fig. 2, panel 1).

Distribution of Severity of Histopathological Lesions According to Expressed HPV ORF. To additionally elucidate differences in the biology of E6 and E7 functions in the estrogen-treated cervix, we applied our CIN grading system to determine the extent of neoplastic progression in transgenic mice expressing the E6 or E7 oncoproteins individually and in combination (Fig. 3). Invasive cancer or high-grade dysplastic lesions (CIN III and CIS) occurred in 100% of K14-E7, K14-E6:E7, and K14-HPV16 transgenic mice, whereas dysplastic lesions in K14-E6 transgenic mice did not progress beyond CIN II (Fig. 3A). Histologically, CIN III and CIS lesions in K14-E7 (Fig. 3, panel 3) and K14-E6:E7 (Fig. 3, panel 4) transgenic mice were similar to those observed in K14-HPV16 mice, except that the degree of nuclear anaplasia and increase in nuclear:cytoplasmic ratio was greater in transgenic mice expressing the entire early region (see Fig. 2, panels 4 and 5). In K14-E6 transgenic mice, the cervical and vaginal squamous epithelium was thicker compared with nontransgenic control mice, with occasional dysplastic cells with increased nuclear size and anaplasia (see arrow Fig. 3B, panel 2). The epithelial-stromal border of K14-E6 transgenic mice was more undulating compared with the linear border in nontransgenic controls; however, the marked papillary Stromal projections of squamous epithelium characteristic of K14-E7 or K14-HPV16 transgenic mice (Fig. 2, panel 3) was absent in K14-E6 transgenic mice. Cancer formation and growth also appeared to distinctively vary according to which HPV ORFs were expressed in the estrogen-treated cervix. Cervical and vaginal cancers in K14-E7 transgenic mice were microinvasive, small, and multifocal (Fig. 3B, panel 5). Cervical malignancies in K14-E6:E7 and K14-HPV16 transgenic mice consisted of multiple nests of invasive squamous cells dispersed over a large area of cervical stromal tissue (Fig. 3B, panel 6). The distinctive biological differences in cancer growth in transgenic mice expressing E7 versus both E6 and E7 support a role for E6 as a late-stage progression factor for cervical cancer in this model.
it has the near equivalent ability of E7 to disrupt squamous epithelial differentiation and cell cycle regulation in the skin.

**Real-time RT-PCR Analysis of E6/E6** Transgene Expression.

One explanation for the lack of neoplastic progression in the K14-E6 transgenic mice could have been “insufficient” transgene expression in cervical epithelium. To determine transgene mRNA expression, quantitative real-time RT-PCR was performed using PCR primers and a TaqMan probe amplifying both full-length and E6 ORF mRNA on total RNA isolated from the lower reproductive tract tissue block (“Materials and Methods”). E6 ORF mRNA expression of homozygous K14-E6 transgenic mice was 13–24-fold elevated compared with heterozygous K14-E7 and HPV transgenic mice (Fig. 5). Development of complete carcinogenesis in K14-E7 and K14-HPV transgenic mice despite a threshold level of transgene expression highlights the cocarcinogenic potency of the E7 oncprotein in conjunction with estrogen in cervical squamous epithelium. Thus, lack of carcinogenic progression in the K14-E6 transgenic mice cannot be explained by insufficient transgene expression.

**Differential Cervical p53 Expression in Transgenic Mice Expressing Either E6 or E7.** Two questions remained that hinged on levels of cervical squamous epithelial p53 protein expression. First, one mechanism for induction of carcinogenesis in transgenic mice expressing E7 oncprotein alone would be coordinate mutation of p53, based in part on previous work demonstrating mutation of p53 in HPV-negative cervical cancer cell lines (45). Second, despite robust expression of transgene mRNA, demonstration of functional activity of the E6 transgene would bolster our findings that lack of neoplastic cervical progression was because of differential oncprotein function rather than differential transgenic protein expression in mouse cervical epithelial cells. In CINIII lesions of K14-E7 transgenic mice, there was a low-level induction of p53 protein expression compared with nontransgenic controls (Fig. 6, panels 3 and 1, respectively), with an additional induction of p53 protein in cervical cancers from this transgenic lineage (Fig. 6, panel 4). However, the intensity of p53 immunostaining in these cancers remained much less than that from islet cell carcinomas derived from transgenic mice expressing SV40 T-antigen behind a rat insulin promoter (Fig. 5, panel 6). These data are consistent with coordinate p53 up-regulation by E7 oncprotein (46), rather than stabilization and accumulation of a mutant p53 protein (47). In marked contrast to p53 induction in K14-E7 transgenic mice, there was no detectable p53 immunostaining in CINII/III lesions in K14-E6 or invasive transformation zone squamous cancers in K14-HPV16 transgenic mice (Fig. 6, panels 2 and 5) consistent with E6-E6AP mediated p53 protein destruction (48, 49).

**Apoptosis in Cervical Carcinogenesis in Transgenic Mice.** As previous work in transgenic lens and retina demonstrated an increase in apoptosis when E7 was expressed alone without E6 (38, 50), we expected a similar biology in the estrogen-induced cervical epithelium of K14-E7 transgenic mice. However, a combination of histopathology and terminal deoxynucleotidyltransferase-mediated nick end labeling assay failed to demonstrate apoptotic cells either at early (4 weeks hormone treatment) or final (24 weeks hormone treatment) stages of estrogen-induced cervical carcinogenesis in transgenic mice of each genotype in this study (data not shown). Induction of apoptosis was also not detected in the cervical squamous epithelium of other genotypes of transgenic mice during cervical carcinogenesis or nontransgenic controls in response to chronic estrogen.

**Centrosome Abnormalities in Cervical Neoplasia and Cancers in Transgenic Mice.** Centrosome abnormalities have been reported previously in a wide array of human cancers including cervical neoplasia (37). In particular, both E6 and E7 viral oncproteins have been shown to induce an increase in centrosome number albeit via...
Fig. 4. Reproductive tract squamous epithelial proliferation in transgenic compared with nontransgenic mice. Reproductive tract squamous epithelial proliferation is incrementally increased over time in K14-HPV16 (n = 6), K14-E7 (n = 4–6), and K14-E6-E7 (data not shown) transgenic mice compared with either K14-E6 (n = 4–8) transgenic counterparts or nontransgenic controls (n = 5–7). A, distribution of proliferative squamous epithelial cells in transgenic and nontransgenic reproductive tract (panels 1, 3, 5, and 7) and ear skin (panels 2, 4, 6, and 8). Nontransgenic (panel 1) and K14-E6 (panel 3) mice demonstrate a similar pattern in the cervical epithelium with proliferating squamous epithelial cells restricted to the basal and immediate suprabasal layers. In contrast, the cervical epithelium of both K14-E7 (panel 5) and K14-HPV16 (panel 7) evidence BrdUrd-positive cells in the upper suprabasal layers and filling papillary projections into the stroma. In nontransgenic skin, rare basal cells are proliferating (panel 2), whereas in each transgenic line (K14-E6, panel 4; K14-E7, panel 6; and K14-HPV16, panel 8) multiple epidermal cell layers are filled with proliferating keratinocytes. The contrast between induction of proliferation in ear and cervix of K14-E6 transgenic mice (panels 3 and 4) is striking. Bar = 20 μm all panels, B.

Discussion

Using our model of estrogen-induced cervical carcinogenesis, we were able to discriminate both discrete biology and distinct functions for the HPV E6 and E7 oncoproteins. The cervix and vagina were exquisitely sensitive to E7 expression by itself. Multiple hyperproliferative, dysplastic lesions were produced, and carcinogenic progression proceeded to invasive malignancies. In contrast, E6 expression alone was unable to affect neoplastic progression, a phenotype that was strikingly different from malignant progression produced in the skin of the same transgenic mice. The fact that centrosome copy number elevations were produced by each HPV oncoprotein individually demonstrates that this potential facet of genomic instability is insufficient for cervical neoplastic progression by itself. Despite a lack of solo carcinogenic potency, E6 was a progression factor in combination with E7, producing larger and more invasive malignancies in double-transgenic mice. As such, both viral oncoproteins are validated as molecular therapeutic targets, with temporal implications for treatment of early versus late-stage lesions in cervical carcinogenesis.

One notable finding in our study was the remarkable potency of the HPV16 E7 oncogene as an estrogen cocarcinogen in the mouse cervix. E7 expression in conjunction with estrogen was sufficient to induce the requisite components of carcinogenic progression including hyperproliferation, inhibition of epithelial differentiation leading to high-grade dysplasia, insufficient compensatory induction of apoptosis, and centrosome copy number elevations (53). Hyperproliferation could be explained by a repertoire of E7 functions shown previously to dysregulate the cell cycle including: E7 binding, destabilization, and consequent destruction of pRB, p107, and p130, sequestration of Mi2 histone deacetylase, induction of c-Myc, and abrogation of transforming growth factor β inhibitory signaling (5). It is important to keep in mind that estrogen itself increased reproductive tract squamous epithelial proliferation (54). Our data suggested that E7 complemented and amplified an underlying proliferative stimulus propagated by the estrogen alone. One molecular explanation for this finding was induction of c-Jun/AP-1 activity by estrogen and HPV16 E7. In particular, HPV16/18 E7 has been shown to increase c-Jun protein levels and transcriptional activity (11), and ligand-bound estrogen receptor has also been shown to activate AP-1 mediated transcription by indirect “tethered” binding to AP-1 complexes on DNA (55). In addition to hyperproliferation, the development of neoplastic progression via high-grade dysplasia and ultimately malig-
nancy in K14-E7 transgenic mice was likely facilitated by a combination of p21 inactivation, which uncoupled squamous epithelial terminal differentiation from proliferation (44), and centrosome copy number dysregulation, which produced multipolar mitotic spindles, abnormal nuclear divisions, and potentially, cellular multinucleation and aneuploidy (52, 56, 57). Estrogen itself could induce direct DNA damage via its catechol metabolites (58–60), which could have been the potential "initiating" step in our model of cervical carcinogenesis.

The absence of concomitant apoptosis, at any stage of cervical neoplastic progression in estrogen-treated K14-E7 transgenic mice, was also surprising. Previous studies demonstrated coordinate induction of apoptosis in both embryonic lens and retina, and the epidermis of transgenic mice with E7 expression targeted to each of these epithelia (38, 50). Whereas induction of apoptosis in embryonic tissue by E7 could have been because of increased sensitivity of developing epithelia to E7 functions, the differential apoptosis in skin compared with cervix merits additional discussion. In the skin, apoptosis was primarily detected in the granular and stratum corneum layers. These epidermal layers contained nucleated cells in the transgenic mice (parakeratosis) compared with anucleate cells in nontransgenic controls (38, 50). As such, apoptosis in the skin model could have been because of persistent E7 expression in keratinocytes, in which the terminal differentiation program was already activated. However, the granular and corneal squamous epithelial cell layers do not exist in the cervix. Therefore, lack of susceptible target cells could be one mechanism underlying the lack of apoptosis in the cervix of estrogen-treated K14-E7 transgenic mice. In addition, the inability to detect apoptosis in the basal layer of cervical squamous epithelium in these same transgenic mice could have been because of paracrine elaboration of survival factors, such as insulin-like growth factor I, epidermal growth factor, platelet-derived growth factor, and transforming growth factor β/α (61–66). These survival factors could have balanced the unopposed expression of E7 in the vaginal and cervical basal squamous epithelial cells of estrogen-treated K14-E7 transgenic mice. Importantly, prior work in the skin has also demonstrated functional p53 in the squamous epithelium of K14-E7 mice. Specifically, coordinate induction of p53 and its target p21 was maintained in the keratinocytes within irradiated transgenic skin (40). As such, the lack of apoptosis in the estrogen-treated

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**Fig. 5.** Transgene mRNA expression levels in the cervico-vaginal epithelium of transgenic mice in response to 1 month of estrogen treatment. Real-time quantitative RT-PCR from mRNA isolated from the lower reproductive tract (see Fig. 1), using PCR primers and a TaqMan probe specific for both full-length and E6 mRNA. The Y axis is relative transgene expression data normalized for the content of endogenous histone 3.3A mRNA. Despite the modest phenotype, homozygous K14-E6 transgenic mice evidence marked transgene expression compared with K14-E7 and K14-HPV16 transgenic counterparts.

**Fig. 6.** Immunohistochemical determination of reproductive tract squamous epithelial p53 protein expression in transgenic and nontransgenic mice. Low-level p53 staining is evident in both basal (arrow and inset) and suprabasal cells (arrow) of nontransgenic mice (panel 1). In contrast, the basal and suprabasal cells (brackets) of K14-E6 transgenic mice lack detectable p53 protein expression (panel 2 and inset). p53 protein expression appears to be coordinately and incrementally induced in K1-E7 transgenic mice in CIN-III (panel 3) and invasive cancers (panel 4). Invasive cancers of K14-HPV16 transgenic mice (panel 5) display a low to moderate level of p53 protein expression, possibly related to a lower level of E6 transgene expression compared with their homozygous K14-E6 counterparts. An islet cell carcinoma from a RIP-TAg transgenic mouse (panel 6) displays marked induction of p53 protein expression because of sequestration by SV40 T antigen. This level of p53 protein detection would be also consistent with stabilized, mutant p53. Bar, all panels = 20 μm.
regarding the relevance of this parameter of genomic instability, our model of cervical carcinogenesis reinforced our previous conclusions that proliferation and differentiation.

Inability of the oncoprotein by itself to affect cervical epithelial cells was insufficient to support neoplastic progression in the face of the clear demonstration that these particular E6 functions by themselves could also have contributed to resistance to their dysregulation despite perturbation by the oncoprotein (7). E6 binding to the calcium-binding protein, E6BP (67, 68), has been shown to inhibit keratinocyte terminal differentiation in cell culture and could have been a potential mechanism for uncoupling terminal differentiation from proliferation. E6 complex formation with either the human homologue of the Drosophila discs-large protein (69) or paxillin (70) could each disrupt basal cell polarity potentially increasing proliferation and facilitating dysplasia by permitting escape of these proliferating cells into the upper stratified squamous epithelial layers. E6-mediated activation of the human telomerase reverse transcriptase component of telomerase (71, 72) could also have contributed to in vivo immortalization of squamous epithelial cells, allowing for persistence of transformed cells and their subsequent progression to malignancy. However, the lack of induction of cervical epithelial cell proliferation and high-grade dysplasia, suggests that either these cellular targets do not interact with E6 in the tissue and hormonal context of this model or that the murine cervix is resistant to their dysregulation despite perturbation by the oncprotein. Certainly the lack of a robust neoplastic phenotype cannot be explained by either lack of reproductive tract E6 expression or even function, as both p53 expression was decreased and centrosome copy number elevated in these transgenic mice. However, our results clearly demonstrated that these particular E6 functions by themselves were insufficient to support neoplastic progression in the face of the inability of the oncprotein by itself to affect cervical epithelial proliferation and differentiation.

Similarly, the discordance in neoplastic potency between HPV E7 and E6 in the face of similar elevations in centrosome number in this model of cervical carcinogenesis reinforced our previous conclusions regarding the relevance of this parameter of genomic instability, per se, as a predictor of ultimate neoplastic progression. Our current model is that centrosome elevations arise by two different mechanisms in E6- or E7-expressing cells (56, 57). In E6-expressing cells, centrosome abnormalities were detected in polynucleated cells, many of which were senescent. These data were consistent with the detection of occasional cells with markedly increased nuclear:cytoplasmic ratio in the cervical epithelium of estrogen-treated K14-E6 transgenic mice (Fig. 3). In contrast, centrosome abnormalities in E7-expressing cells were detected in proliferative diploid cells before additional features of genomic instability (57), suggesting that E7 itself is an independent driving force for the induction of genomic stability in cells with the potential for neoplastic progression. The current study is the first determination that this concept of parallel alteration in centrosome number control indeed occurs in the cervix expressing either HPV oncogene and that these centrosome abnormalities are associated with different neoplastic consequences.

However, our results with individual expression of E7 or E6 in the estrogen-treated cervix contrasted sharply to that seen in the skin of the same transgenic mice. In skin, both E7 and E6 were potent inducers of hyperproliferation (Fig. 4; Refs. 32, 33). Both oncogenes dysregulated terminal differentiation and allowed escape of proliferating cells to the upper epidermal layers. Most strikingly, the carcinogenic potential of these oncogenes was reversed in skin. There, E7, alone or in combination with two-stage chemical carcinogens, induced predominantly benign papillomas, possessing a low frequency of malignant conversion (32, 39), whereas epidermal cancer was the predominant lesion in transgenic mice expressing E6 alone (33) or in combination with chemical carcinogens (39). Additional examination of the data from these experiments also supported a role for E6 as a late-stage malignant progression factor in epidermis, reminiscent of our current results in the mouse cervix, albeit when both HPV oncogenes were coexpressed. In skin, the combination of E6 and chemical carcinogens increased the frequency of spindle-cell compared with well-differentiated epidermal carcinomas. Spindle-cell cancer is a more advanced stage of malignant progression in mouse skin carcinogenesis (73). Despite this similarity, the overall discordance in solo carcinogenic potency of E7 and E6 in mouse skin and cervix highlights the importance of cell-type, tissue, and hormonal context in the modeling of human disease by transgenic models.

However, despite our compelling demonstration of distinctive E7 and E6 functions in the mouse cervix, these viral oncogenes are not drivers of cervical carcinogenesis. Our results with individual expression of E7 or E6 in the estrogen-treated cervix contrasted sharply to that seen in the skin of the same transgenic mice. In skin, both E7 and E6 were potent inducers of hyperproliferation (Fig. 4; Refs. 32, 33). Both oncogenes dysregulated terminal differentiation and allowed escape of proliferating cells to the upper epidermal layers. Most strikingly, the carcinogenic potential of these oncogenes was reversed in skin. There, E7, alone or in combination with two-stage chemical carcinogens, induced predominantly benign papillomas, possessing a low frequency of malignant conversion (32, 39), whereas epidermal cancer was the predominant lesion in transgenic mice expressing E6 alone (33) or in combination with chemical carcinogens (39). Additional examination of the data from these experiments also supported a role for E6 as a late-stage malignant progression factor in epidermis, reminiscent of our current results in the mouse cervix, albeit when both HPV oncogenes were coexpressed. In skin, the combination of E6 and chemical carcinogens increased the frequency of spindle-cell compared with well-differentiated epidermal carcinomas. Spindle-cell cancer is a more advanced stage of malignant progression in mouse skin carcinogenesis (73). Despite this similarity, the overall discordance in solo carcinogenic potency of E7 and E6 in mouse skin and cervix highlights the importance of cell-type, tissue, and hormonal context in the modeling of human disease by transgenic models.
invariably coexpressed in human cervical cancer. Both our current and prior work is concordant with a role for E6 as a late-stage malignant progression factor in both cervix and skin of double E6/E7 transgenic mice (39). In the cervix, E6 and E7 coexpression contributes to the formation of large, extensively invasive cancers in double-transgenic mice, and the enhanced chromosomal copy number dysregulation in the malignancies in these mice compared with counterparts expressing E7 alone.

In summary, this study highlights the distinctive functions of the E7 and E6 oncoproteins in cervical carcinogenesis with the caveat that several species-specific and technological differences exist between our model and human cervical neoplastic or malignant disease. Collectively, our data suggest that both induction of squamous epithelial proliferation and perturbation of centrosome copy number contribute to progression to invasive cervical cancer. Moreover, whereas E6 appears to have a low neoplastic penetrance alone, it facilitates the genesis of aggressively malignant cervical cancers in conjunction with E7. Future work will genetically dissect discrete E7 and E6 oncoprotein functions to elucidate the mechanisms underlying their cooperation in cervical carcinogenesis.

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E6 AND E7 IN CERVICAL CARCINOGENESIS


Dissection of Human Papillomavirus E6 and E7 Function in Transgenic Mouse Models of Cervical Carcinogenesis

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